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In Vitro Hemolytic Potency and Binding of Chlorpromazine to Plasma Protein and Erythrocytes of Rat and Dog (38579)

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The tranquilizers of the phenothiazine group have been demonstrated to affect the hemolytic process. Freeman and Spirtes (1) showed that chlorpromazine (CPZ) and prochlorperazine, *in vitro*, had a strong hemolytic effect at higher concentrations but antihemolytic at lower. Chaplin *et al.* (2) reported that phenothiazine derivatives had both hemolytic and antihemolytic effects on stored blood. It was later demonstrated that CPZ prolonged the total hemolysis time of dog and human blood in 0.3 *M* glycerol (3). Giri and Peoples (4) reported that CPZ at 10^{-3} *M* concentration caused the 50% hemolysis of the rat whole blood much faster than that of dog. The present investigation was carried out in an attempt to explain the striking difference between the rates of CPZ-induced hemolysis of rat and dog red cells, by employing the pharmacodynamic principles of drug action. While drugs in the body exist in the bound and/or free states, their pharmacological action depends upon the availability of the free form. The portion of the drug bound to the plasma proteins or to any of the tissue constituents is inactive pharmacologically but serves as a reservoir for the free form. It was envisioned that the slower rate of hemolysis of dog blood in response to CPZ could be attributed to a greater percentage of its binding to the erythrocytes. This report demonstrates that a greater percentage of CPZ binding to dog erythrocytes might be partially responsible for a slower rate of hemolysis as compared to rat erythrocytes which bind CPZ to a lesser degree.

Materials and Methods. Male Sprague–Dawley rats weighing 300–350 g were purchased from Simonsen Laboratory Incorcorporated, Gilroy, CA, and conditioned Beagle dogs from Animal Resources Service at UC Davis. The chlorpromazine hydrochloride was a generous gift from Smith, Kline and French Labs, Philadelphia.

Rats were sacrificed by decapitation and blood was collected in an Erlenmyer Flask containing either EDTA (1 mg/ml blood) for binding, or heparin (50 units/ml) for hemolysis studies. Dog blood was collected by venipuncture using anticoagulants as above. The hematocrit in each case was determined immediately using the conventional hematological technique. The rest of the blood sample was centrifuged at 2000 rpm for 15 minutes (International Centrifuge, Model UV). The plasma was aspirated and its osmolarity and protein content determined using an osometer (Advanced Inst. Inc.) and a refractometer (5) respectively. The plasma was then diluted in a ration of 1–3 with 0.9% buffered NaCl solution, pH 7.081 (6). The red cells were washed twice using the buffered NaCl solution and finally suspended in the same buffer to three times the original blood volume.

Binding studies were carried out using the equilibrium dialysis technique (7). The dialysis bags contained either 2 ml of the diluted plasma or diluted RBC suspension. They were immersed in 30 ml beakers each of which contained a different amount of CPZ in a total volume of 18 ml buffered NaCl (0.9%, pH 7.086). Beakers were placed in a shaker and dialysis was carried out in a cold room at 4° with gentle shaking for a period of 16 hr. Plasma or red cells blanks were run as above except that they were dialyzed against 18 ml of buffered NaCl free of CPZ. Internal standards were run simultaneously at each concentration of CPZ using 2 ml of buffered NaCl solution in the dialysis bag. This minimized any errors arising from the binding of the drugs by the dialysis bags. At the end of dialysis the concentration of CPZ in the external solution was analyzed spectrophotometrically at 255 nm (3) using a modified Beckman Gilford spectrophotometer and quartz cuvettes with a 1 cm light path.

The 50% hemolysis time (TH50) was determined using whole blood of rat and dog, rat red cells suspended in dog plasma and dog red cells suspended in rat plasma. A 0.5 ml aliquot of heparinized blood from rat or dog was diluted to 50 ml with buffered NaCl solution. A 5 ml sample of rat or dog blood was centrifuged for 15 min at 2000 rpm. The plasma was aspirated and the red cells washed twice in buffered NaCl solution. The rat red cells were suspended in the entire volume of dog plasma and the dog red cells were suspended in the rat plasma. Reconstituted blood samples were diluted with buffered NaCl solution in the same manner as the whole blood.

The effects of CPZ ($10^{-3}M$) on 50% hemolysis time was determined according to the method described elsewhere (4) with the modification that 1.5 ml of diluted blood (1:100) and 1.5 ml of CPZ ($2 \times 10^{-3}M$) solution made in 0.9% buffered NaCl, pH 6.85, were used in the cuvette. Control experiments using NaCl solution made in the same buffer as CPZ and in equal osmolarity to CPZ were run in the same way to eliminate the effect of osmolarity in determining the TH50 values of whole blood or red cell suspension. Under these conditions, no hemolysis was detected for as long as 60 min.

Data are reported in terms of mean values ± standard error of the mean (SE). The Student's t test was applied to test the significance of the differences between means.

Results and Discussions. The percent of CPZ bound to plasma protein and erythrocyte of rat and dog is summarized in Table I. Dog plasma protein appeared to bind CPZ to the same extent as the rat plasma protein. Although, dog plasma protein was found to bind CPZ slightly more than that of the rat, but this difference in binding between the two was not statistically significant.

There was a highly significant increase in CPZ binding by dog erythrocytes over those of the rat. The differences at CPZ concentrations of $3.5 \times 10^{-5}M$ and $1.75 \times 10^{-5}M$ were 120% and 150% respectively. The unique property of dog red cells that enables a higher percentage of CPZ binding cannot be explained by a difference in the hematocrit, as no statistically significant difference was noticed in hematocrit between the dog and rat (Table II).

Effects of CPZ at $10^{-3}M$ concentration on TH50 values of whole blood, red cells suspended in buffered saline, rat red cells suspended in dog plasma and dog red cells in rat plasma are summarized in Table III. CPZ at $10^{-3}M$ concentration produced hemolysis of both rat and dog red cells. The rat red cells were more sensitive to this effect of CPZ than that of the dog. The hemolytic effect of CPZ at higher concentrations is consistent with our earlier finding (4) as well as with the finding of other investigators (1, 2). The exact mechanism for the hemolytic effect of CPZ is not clearly understood. We feel however that this is the result of a direct action of CPZ on the red cell membrane. This hypothesis is supported in part by the findings of Greig and Gibbons (8) that phenothiazine drugs increase the hemolytic potency of agents (saponin, digitonin and sodium oleate) which act directly on the red cell membrane.

The 50% hemolysis time in response to CPZ on the rat whole blood or rat red cells suspended in buffered NaCl solution was much shorter than on the counterpart red cells of the dog. This could be attributed in part to a lesser degree of CPZ binding by the red cells of the rat. This would certainly allow the availability of a greater percentage of free drug to interact with the active sites of erythrocytes and thereby initiate the hemolytic process much sooner in the rat than in the dog.

An alternative explanation is that dog red cells contain two types of receptors for CPZ binding: The silent receptors and the active receptors. The interaction of CPZ molecules with the former results in soaking up the drug molecules with no effect, while interaction with the latter type triggers the hemolytic processes. Thus, the binding of CPZ molecules to the silent receptors would tend to decrease the availability of the free form of the drug molecules at the active site.

In rats however, if the silent receptors are absent from the erythrocytes, the free form of the drug would tend to concentrate at the active site. This might explain why CPZ at $10^{-3}M$ concentration takes longer to hemolyze 50% of dog red cells in whole blood or

TABLE I. Binding of Chlorpromazine (CPZ) at Different Concentrations with the Plasma Protein (PP) and Red Cells of the Rat and Dog.[a]

	Percent of CP2 bound at two different Concentrations, AVG ± SE	
	$3.5 \times 10^{-5}M$	$1.75 \times 10^{-5}M$
Rat PP	41 ± 3.2 (5)[b]	50.8 ± 7.5 (5)
Dog PP	50 ± 4.1 (5)	61.4 ± 4.1 (5)
P value between rat and dog	NS[c]	NS[c]
Rat red cells	23.6 ± 4.3 (5)	26.2 ± 7.5 (5)
Dog red cells	53.1 ± 1.9 (5)	65.4 ± 5.8 (5)
P value between rat and dog	<0.001	<0.01

[a] Equilibrium dialysis was used to determine the binding. See the Method Section for details.
[b] Figures in parenthesis are the number of animals used.
[c] Not significant.

TABLE II. Hematocrit, Plasma Osmolarity and Plasma Protein of Rat and Dog Blood.

Animals	Hematocrit Avg ± SE	Osmolarity in milliosmal/kg Avg ± SE	Plasma protein in 100 ml Avg ± SE
Rat	43.8 ± 1.3 (8)[a]	298 ± 0.9 (4)	6.2 ± 0.1 (15)
Dog	54.4 ± 1.1 (8)	303.9 ± 4.5 (4)	5.9 ± 0.1 (10)
P value between rat and dog	NS[b]	NS[b]	NS[b]

[a] Figures in parenthesis are the number of animals used.
[d] Not significant.

TABLE III. *In Vitro* Effect of Chlorpromazine on 50% Hemolysis Time (TH50) of Rat and Dog Red Cells.[a]

Red cells	50% Hemolysis time in seconds in response to CPZ $10^{-3}M$ Avg ± SE	*P* Value between rat and dog
Rat whole blood	68 ± 3.8 (5)[b]	
Dog whole blood	320 ± 24 (5)	<0.001
Rat RBC in buffered NaCl solution	49.5 ± 6.1 (4)	
Dog RBC in buffered NaCl solution	145.5 ± 16.5 (4)	<0.01
Rat RBC + dog plasma[c]	92.4 ± 13.9 (5)	
Dog RBC + rat plasma[d]	154.5 ± 13.5 (4)	<0.05

[a] See the Method Section for red cell preparation and determination of TH50.
[b] Figures in parenthesis are the number of animals used.
[c] *t* value between rat whole blood and rat RBC + dog plasma is significant at 10% level.
[d] *t* value between dog whole blood and dog RBC + rat plasma is significant at 1% level.

suspended in buffer than that of the rat red cells under the same experimental conditions. Although the presence of silent receptors on the dog red cells and their absence from the rat is purely speculative, the concept is by no means new. Various investigators (9) have employed the silent receptor concept as a possible mechanism for the lack of drug action on different tissues under varying experimental conditions.

It does not mean however, that the authors have any intention to rule out other possibilities that might account for the difference in the rate of CPZ-induced hemolysis of rat and dog red cells. For example, it is conceivable that dog red cell surface differs from that of rat in the sense that it may need a greater concentration of CPZ molecules to cause a type of disturbance on the surface sufficient to cause hemolysis. Secondly, a greater percentage of CPZ binding to dog red cells as compared to rat perhaps represents entrance of CPZ into the red cell. Thirdly, dog red cells might be mechanically more sound to resist the CPZ-induced hemolysis than that of the rat. The verification of these and other possibilities have to wait until further experiments are carried out.

The 50% hemolysis time of rat red cells suspended in dog plasma was higher than that of rat whole blood. The 50% hemolysis time of dog red cells suspended in rat plasma was lower than that of dog whole blood. This again suggests a greater ability of the dog plasma to bind CPZ molecules than that of the rat plasma. A difference in the 50% hemolysis time of red cells suspended in the heterologous plasma can not be attributed to plasma osmolarity, as no difference in this value was found between the dog and rat plasma (Table II).

Summary. It was demonstrated by equilibrium dialysis that dog red cells have a greater ability to bind chlorpromazine than rat red cells. Dog plasma was shown to have a greater ability to bind chlorpromazine than rat plasma although this difference was not statistically significant. In the presence of CPZ ($10^{-3}M$), the 50% hemolysis time of dog red cells suspended in homologous plasma or in 0.9% buffered NaCl solution was much greater than that of rat red cells treated in the same manner. The 50% hemolysis time of dog red cells suspended in homologous plasma was considerably shortened when they were suspended in rat plasma. Conversely, an increase in the 50% hemolysis time was obtained when rat red cells were suspended in dog plasma. Several possibilities for the slower rate of CPZ-induced hemolysis of dog red cells as compared to rat has been discussed in the present report.

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